Effect of Plating Medium on Heat Activation Requirement of *Clostridium botulinum* Spores

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Clostridium botulinum 62A and ATCC 25763 spores required heat activation for maximum colony formation when plated on reinforced clostridial agar (BBL Microbiology Systems) but not when plated on botulinum assay medium. Spores from strains B-aphis and 53B did not exhibit heat activation when plated on either medium.

Heat activation of clostridial spores was first reported by Reynolds and Lichtenstein (H. Reynolds and H. Lichtenstein, Abstr. Annu. Meet. Soc. Am. Bacteriol. 1949, G-7, p. 9) and has remained an elusive phenomenon which can be affected by the composition of the sporulation medium (9, 10), the time and temperature of the heat treatment (7), the length and temperature of plate incubation (4), and interstrain variation (7).

In evaluating the efficacy of several plating media for *Clostridium botulinum*, I found that the composition of the plating medium also affected the expression of heat activation. These observations are reported herein.

C. botulinum strains 62A, ATCC 25763 (also type A), B-aphis, and 53B were obtained from C. N. Huhtanen, Eastern Regional Research Center, U.S. Department of Agriculture, Philadelphia, Pa., and maintained in cooked meat medium (Difco Laboratories).

Spores were prepared by inoculating 10 ml of broth (5% Trypticase [BBL Microbiology Systems]-0.5% peptone-0.125% K_2HPO_4-0.075% $NaHCO_3$) (1) with a loopful of cooked meat medium culture. After 4 days of incubation at 30°C, 2 ml of the broth was transferred into 200 ml of fresh broth. After 21 days of incubation at 30°C, 50 to 70% sporulation occurred. The spores were cleaned by differential centrifugation (8) and suspended to a final volume of 25 ml in sterile distilled water. Microscopic examination of this final spore preparation in a Petroff-Hausser counting chamber (Hauser Scientific) under phase-contrast optics showed that strains 62A, ATCC 25763, B-aphis, and 53B contained 2.3×10^9, 2.7×10^9, 1.8×10^9, and 4.5×10^8 refractile spores per ml, respectively. Undiluted preparations had <0.1% phase-dark spores, no vegetative cells, and little debris.

All plating, incubation, and counting procedures were done in an anaerobic chamber (Coy Laboratory Products) similar to that described by Aranki et al. (2) and charged with a gas mixture containing 90% N_2, 5% CO_2, 5% H_2, and <5 ppm of O_2. Media and diluents were prereduced in the chamber for at least 48 h before use.

Spores were heat shocked by adding 0.1 ml of spore suspension to 9.9 ml of sterile distilled water preequilibrated to 80°C. After 10 min at 80°C, 1.0 ml was transferred to 9.0 ml of cool, sterile diluent (0.1% peptone [Difco]). Non-heat-shocked spores were similarly diluted but not heated. Serial dilutions were prepared, and 0.1 ml from a given dilution tube was spread plated onto reinforced clostridial agar (RCA; BBL Microbiology Systems) or the botulinum assay medium (BAM) of Huhtanen (6), which contained 0.5% yeast extract, 0.265% nutrient broth, 0.2% glucose, 0.05% sodium thioglycolate, 0.5% tryptone (all from Difco), and 0.12% K_2HPO_4. Agar was added to both RCA and BAM to a level of 2%. Colonies were counted after 3 days of incubation at 30°C; no additional colonies appeared if the incubation was extended to 7 days.

Representative results are shown in Table 1. For strains 62A and ATCC 25763, heat activation (manifested as increased ability to form colonies) was demonstrated on RCA but not on BAM. Heat-shocked spores of strain B-aphis consistently showed a slight decrease in number on both media, suggesting that these spores are more heat sensitive than spores from the other strains. Although the above results were consistent in all experiments, spores from strain 53B showed a variable response to heat shock, with little, if any, heat activation. Counts on BAM were reproducibly higher than counts on RCA for this strain. For all strains, the number of heat-shocked spores on RCA was always less

TABLE 1. *Effect of heat shock on C. botulinum spores plated on BAM or RCA*

Strain	Plating medium	Heat shock[a]	CFU[b]/ml	Plating efficiency (%)[c]	Heat activation[d]
62A	BAM	+	1.8×10^9	78	1.0
	BAM	−	1.7×10^9	77	
	RCA	+	1.2×10^9	52	4.6
	RCA	−	2.6×10^8	11	
ATCC 25763	BAM	+	1.1×10^9	41	1.3
	BAM	−	8.6×10^8	32	
	RCA	+	6.5×10^8	24	4.0
	RCA	−	1.6×10^8	6	
B-aphis	BAM	+	7.9×10^8	43	0.8
	BAM	−	9.5×10^8	52	
	RCA	+	8.4×10^8	46	0.9
	RCA	−	9.5×10^8	52	
53B	BAM	+	3.9×10^8	86	1.4
	BAM	−	2.8×10^8	62	
	RCA	+	1.4×10^8	31	1.3
	RCA	−	1.1×10^8	24	

[a] At 80°C for 10 min.
[b] CFU, Colony forming units.
[c] [(CFU/ml)/microscopic count] × 100.
[d] (Heat-shocked CFU/ml)/(non-heat-shocked CFU/ml).

than the number of non-heat-shocked spores on BAM. This indicates that BAM obviates the requirement for heat activation.

Heat activation of *Clostridium perfringens* spores on RCA is also strain dependent (7). Roberts and Ingram (9) reported that heat activation of *C. botulinum* type B spores plated on RCA ranges from 0 to 15-fold, depending on the sporulation medium used. Rowley and Feeherry (10) demonstrated that *C. botulinum* 62A spores exhibit heat activation in a chemically defined medium.

The component(s) of BAM responsible for its high plating efficiency in the absence of heat activation is unknown. BAM and RCA both contain yeast extract, which is beneficial to spore germination (3, 11, 12). The addition of tryptone to RCA does not change its plating efficiency (unpublished data). Although Treadwell et al. (11) have suggested that sodium thioglycolate may be inhibitory to spore germination, this appears not to be the case with BAM, as plating efficiencies of 100% were occasionally obtained. Fortification of the plating medium with calcium dipicolinic acid eliminates the heat activation requirement of *Bacillus subtilis* spores (4).

Levinson and Feeherry (7) have suggested that storage for 1.5 months at 25°C might be sufficient to activate *C. perfringens* spores. My experiments were conducted within 1 month after the initial preparation of fresh *C. botulinum* spore crops and yielded consistent results during this period. When spores from strain 62A were plated after 4 months of storage at ambient temperature, the expression of heat activation was greatly reduced (data not shown). Forty percent of strain 53B spores became phase-dark (i.e., germinated) after 4 months of storage. These observations suggest that some form of "storage activation" does occur with *C. botulinum* spores and that careful consideration should be given to conditions of spore storage during the course of heat activation studies.

In addition to obviating the heat activation requirement of the four strains studied, BAM allows preliminary counts to be made after 24 h of incubation, with no secondary colonies appearing after 3 days. In limited studies with strain 62A spores plated on modified McClung and Toabe egg yolk agar (5) and anaerobic agar (Difco), I found that at least 48 h of incubation was required for colony formation and that secondary colonies, which increase the plate counts, could appear at as late as 6 days of incubation. For these reasons, BAM is now the medium of choice for plating unstressed spores from established strains of *C. botulinum* in this laboratory.

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